EMBO
Molecular Medicine

Manuscript EMM-2011-01047

Early neutralizing IgG response to Chikungunya virus in infected patients targets a dominant linear epitope on the E2 glycoprotein

Yiu-Wing Kam, Fok-Moon Lum, Teck-Hui Teo, Wendy W.L. Lee, Diane Simarmata, Sumitro Harjanto, Chong-Long Chua, Yoke-Fun Chan, Jin-Kiat Wee, Angela Chow, Raymond T.P. Lin, Yee-Sin Leo, Roger Le Grand, I-Ching Sam, Joo-Chuan Tong, Pierre Roques, Karl-Heinz Wiesmüller, Laurent Rénia, Olaf Rötzschke, Lisa F.P. Ng

Corresponding author: Lisa Ng, Singapore Immunology Network

Review timeline:

Submission date:	10 November 2011
Editorial Decision:	13 November 2011
Appeal:	14 November 2011
Editorial Correspondence:	14 November 2011
Author Correspondences:	16 November 2011
Editorial Decision:	09 December 2011
Revision received:	20 December 2011
Accepted:	04 January 2012

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 13 November 2011

Thank you for the submission of your manuscript "Early neutralizing IgG3 response to Chikungunya virus in infected patients targets a dominant linear epitope on the E2 glycoprotein". I have now had the opportunity to carefully read your paper and the related literature and I have also discussed it with my colleagues. I am afraid that we concluded that the manuscript is not well suited for publication in EMBO Molecular Medicine and have therefore decided not to proceed with peer review.

In this manuscript, you report on the identification of a dominant linear B-cell epitope present at the N-terminal of E2 protein of Chikungunya viruses and that is targeted by early neutralizing (in vitro) and protective (in vivo) IgG3 antibodies. The manuscript under consideration at another journal already describes IgG3 as the dominant neutralizing antibodies in response to CHIKV. Yet, we appreciate that the epitope described here is novel and critical residues are identified. However, while this epitope clearly sticks out of the 3D-protein structure likely for a reason, the functional meaning remains to be determined. You further show that the same epitope is nicely recognized by patients across different cohorts and even by non-human primates, which indeed could be exploited for sero-diagnostic of early CHIKV infection. While pre-clinical vaccine trial using the epitope is promising, we also have to realize that many other vaccines have been trials, even in phase I and II. Therefore, all in all, we are not persuaded that your study provides sufficient insight to be further considered in EMBO Molecular Medicine.

I am sorry that I could not bring better news this time but hope that this negative decision does not prevent you from considering our journal for the publication of your future studies.

We look forward to evaluate your next manuscript. You can contact me to discuss any work that you might like to submit informally before submission if you prefer.

Yours sincerely,

Editor
EMBO Molecular Medicine

Appeal 14 November 2011

Since I am a co-author on this manuscript, I take the liberty to write to you to contest some of your arguments which are, sorry to say, not substantiated.

We sent the manuscript to EMBO Mol Med because our work is really translationnal, with an emphasis on Molecular Medicine, entirely your scope, unless you changed scope (I read the article of Philippe Sansonnetti on the goal of this journal, and that's what motivated our decision).

"In this manuscript, you report on the identification of a dominant linear B-cell epitope present at the N-terminal of E2 protein of Chikungunya viruses and that is targeted by early neutralizing (in vitro) and protective (in vivo) IgG3 antibodies. The manuscript under consideration at another journal already describes IgG3 as the dominant neutralizing antibodies in response to CHIKV."

The main results of this other paper is too show that early IgG3 production are associated significantly and strongly with protection against the pathology induced by the CHIKV infection. We did not characterize at that time the response because it would have diluted the message.

"Yet, we appreciate that the epitope described here is novel and critical residues are identified. However, while this epitope clearly sticks out of the 3D-protein structure likely for a reason, the functional meaning remains to be determined."

Dear editor, there is a confusion here. Anybody recognize structures/epitopes that are exposed. This epitope may be in a region that no have function at all. For example, in malaria some antibodies against the Duffy binding proteins recognized epitope outside the structure are involved in the binding to the receptor, and they just provoke a steric hindrance.

"You further show that the same epitope is nicely recognized by patients across different cohorts and even by non-human primates, which indeed could be exploited for sero-diagnostic of early CHIKV infection."

Yes and more over because of our other article (which is is nearly accepted, reviews for the reviewers can be sent to you if you request them), it now can be used as a marker to predict which patients will develop or not later arthralgia (which can last up to a year, when the virus is no more present in the blood, since the viremia last then a week). So this has a real translational application.

"While pre-clinical vaccine trial using the epitope is promising, we also have to realize that many other vaccines have been trials, even in phase I and II."

Dear editor, this argument is not valid. In the malaria field, we had more than 200 trials in Phase 2-3 for all antigens (except maybe MSP3), it is nearly impossible to say which epitopes are linked with protection.

All CHIKV vaccine trials so far have used whole viruses, pseudo viruses (with some success) and DNA constrcuts, but they have no clue what epitopes are targeted and how they can improved the design of the vaccine.

This work is the first which provide a rationale to develop a vaccine based on careffully characterized epitope.

"Therefore, all in all, we are not persuaded that your study provides sufficient insight to be further considered in EMBO Molecular Medicine. I am sorry that I could not bring better news this time but hope that this negative decision does not prevent you from considering our journal for the publication of your future studies. We look forward to evaluate your next manuscript. You can contact me to discuss any work that you might like to submit informally before submission if you prefer."

Thanks for the offer.

Regards

Editorial Correspondence 14 November 2011

Thank you for contacting me.
First let me say that I never mentioned that the paper was out of scope, as it clearly is not. Indeed, I do recognize the translational nature of this work.

I hope you understand that precisely with the previous paper almost accepted and already describing the IgG3 as the main neutralizing antibodies, the novelty factor becomes reduced. I also would like to point out that the vaccine results presented here only present limited protection and viremia reduction, and other described pre-clinical vaccines seem to provide a better efficiency.

Nevertheless, I do understand your deception, and arguments, and to be fair to your paper I will ask for additional advice on it.

This may take a few days, so please be patient and I will contact you again, as soon as we will make a decision.

With my best wishes,

Editor

EMBO Molecular Medicine

Author Correspondence 16 November 2011

Further to the earlier correspondence below. I wish to drop a note that the previous paper has just been accepted, further emphasizing the importance of the story submitted to EMBO Molecular Medicine. Thank you.

Best regards,

Author Correspondence 16 November 2011

Dear editor,

"I hope you understand that precisely with the previous paper almost accepted and already describing the IgG3 as the main neutralizing antibodies, the novelty factor becomes reduced."

I understand the novelty concept but the scope of this paper is the epitope characterization. AND the novelty that the response at that time is mainly focused to a single dominant epitope.

"I also would like to point out that the vaccine results presented here only present limited protection and viremia reduction, and other described pre-clinical vaccines seem to provide a better

efficiency."

This experiment is a proof of concept of the relevance of the epitope for protection. Now we have a rationale for designing a better immunogen and more importantly a method (using the ELISA) to assess it.

2nd Editorial Decision 09 December 2011

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript.

As you will see in the reports below, all three referees find your work of interest. Nevertheless, Ref. #1 is recommending to perform extra controls. In addition, some proof-reading is required, as well as extra discussion on the specific points raised by the referees.

Given these evaluations, I would like to give you the opportunity to revise your manuscript, with the understanding that the referees' concerns must be fully addressed within the time constraints outlined below and that acceptance of the manuscript would entail a second round of review.

Please note that that it is our journal's policy to allow only a single round of revision, and that acceptance or rejection of the manuscript will therefore depend on the completeness of your response and the satisfaction of the referees with it.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension is obtained from the editor. Also, the length of the revised manuscript may not exceed 60,000 characters (including spaces) and, including figures, the paper must ultimately fit onto optimally ten pages of the journal.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

The work here has been well done and presented. The relevance is that the authors examine a human cohort to identify the dominant epitope that the neutralizing response is directed at. The potential for using this epitope as a diagnostic and possibly a subunit vaccine is significant.

Referee #1 (Other Remarks):

Kam and colleagues have analyzed the early neutralizing antibody response in human patients to the alphavirus Chikungunya. Using a pooled set of 30 serum samples from Chikungunya virus-infected patients, the authors demonstrate that the early immune response is dominated by IgG3 antibodies directed against a single linear epitope found at the N-terminal end of the E2 glycoprotein. Removal of these antibodies from the serum resulted in a decrease in the Chikungunya-specific antibody titer. The value of this epitope as a potential diagnostic marker was demonstrated in nonhuman primates as well as in different patient cohorts. The study suggests that this epitope may prove useful in diagnostics as well as in the design of Chikungunya virus vaccines.

Specific comments:

A major concern running through some of the manuscript is the lack of appropriate controls. This is

highlighted in the experiments shown in figures 5, 7, and 8. The authors need to demonstrate the use of control peptides such as a randomized E2EP3 peptide and a peptide from region of E2 that had little or no reactivity with the sera as shown in Figure 2.

Page 13, line 1(Fig 8A): Using the E2EP3 peptide to immunize mice only produced a modest reduction in infectivity of Chikungunya as measured in vitro. The authors should discuss how this compares with other immunogens and their ability to generate neutralizing antibodies.

Page 14, line 12: The two sentences here are incongruent with one another. In the first sentence the authors refer to E2 as the only one of the three surface proteins that reacted with patient sera. In the second they say that neither capsid nor E1 proteins reacted in Westerns. However, capsid is not the third of the surface proteins and thus these two lines do not follow one another, or at the very least are confusing.

Referee #2 (Comments on Novelty/Model System):

The study Kam et al present is complete ane elegant. The models used are the appropriate and the findings clear.

Referee #2 (Other Remarks):

In their manuscript Kam et. al. present novel findings concerning the adaptive immune response against a medically important arbovirus, Chikungunya (CHIKV). Their study is complete and thorough and in my opinion promotes the field since it is adding new knowledge on the antibody response mechanisms against CHIKV, an area poorly understood.

I only have minor points which I would however like to be addressed in the manuscript. These are:

1. In the title Early neutralizing IgG3 I believe 3 should be removed. The authors explain that in detail in the manuscript.
2. In the abstract, E2EP3-specific antibodies are neutralizing and their removal....up to 80%. This sentence is incomplete I think the word antibody should appear after CHIKV-specific.
3. Page 6: Furin E2/E3-cleavage site that...the verb is is missing here.
4. Page 10: between parentheses E2EP3-specific IgG3 was; I believe this should read were to be in accordance with the tense used in the first sentence of the 3rd paragraph of page 15,
5. Page 15: We have previously showed; the verb should be changed to shown. Also since the article they are referring to is not yet published the word previously is not valid.
6. Page 16: The authors discuss that early detection would allow more cost-effective patient management. I believe this is not the case since: a) they detect antibody at day 10 after the onset of symptoms when infectious virus is no longer detectable and b) the treatment for CHIKV is very similar to this used for other arboviruses and aiming only to relieve the symptoms.
7. Page 24: Animals were bled and observed daily for one week than...This should read then.
8. Figure 1. panel B is not required the western blots in both Figure 1 and Figure S1 are very clear.
9. Figure 5 panel D, When antibodies were depleted infectivity of the virus went only up to approx 50%. Could the authors comment on that?

Referee #3 (Comments on Novelty/Model System):

two appropriate and complementary models were used: human convalescent sera and murine infections

Referee #3 (Other Remarks):

In this manuscript, Lisa Ng's lab and collaborators have studied blood samples from ~30 convalescent people following the mosquito-borne Chikungunya virus infection. They determined that neutralizing antibody rapidly appears, and that this is of the IgG3 subclass. Further, they mapped the neutralizing epitope to an N-terminal conserved linear superficial peptide of the E2 glycoprotein (which they called E2EP3 stkdfnvykatrpylah), located the peptide on the 3D structure

of the E2 gyycoprotein. They determined crucial amino acids for the Ab binding of the peptide. Then, they extended the study by immunizing mice with virus and elicited anti-E2EP3 peptide Abs and finally vaccinated mice with the peptide-KLH, producing protection from arthritis (ankle swelling) following experimental infection.

The work is carefully and thoroughly done, presented in an elegant and compelling manner. It is highly likely this study will result in the development of a vaccine for people in the endemic Indian Ocean region, since eradication of the vector is impossible.

I would recommend one very minor modification to the manuscript: IgG3 is one of 2 Ab subclasses that are readily transmitted across the placenta. The paper's authors could add one sentence about the protection of newborns from infection via maternal immunization.

1st Revision - Authors' Response 20 December 2011

We thank the reviewers for their positive, encouraging and valuable feedback. We have responded to all the pointers and queries raised by all referees very carefully and have revised the manuscript incorporating all their suggestions to improve the quality of the manuscript (with changes shaded in yellow for easy reference). To highlight our responses to their queries and comments, we provided all the referees' text (in blue) and our point-wise replies accordingly below.

Referee #1:

(Comments on Novelty/Model System):

The work here has been well done and presented. The relevance is that the authors examine a human cohort to identify the dominant epitope that the neutralizing response is directed at. The potential for using this epitope as a diagnostic and possibly a subunit vaccine is significant.

Response: We thank the referee for this positive comment on the quality of our manuscript.

(Other Remarks):

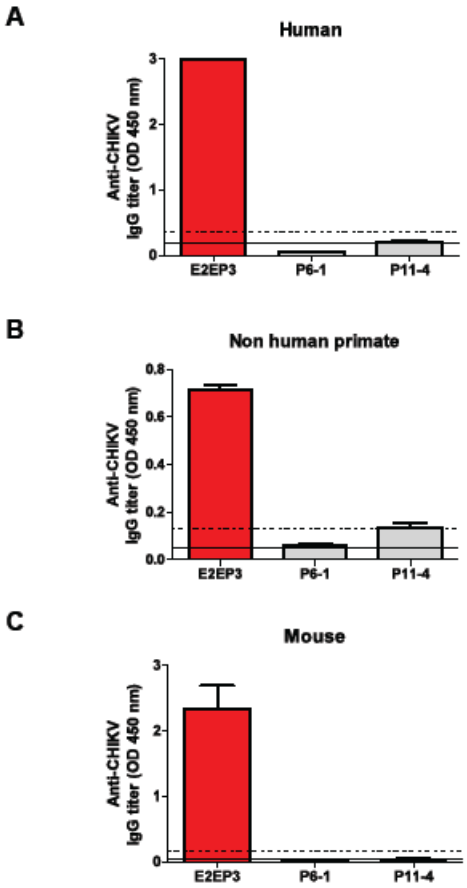
Kam and colleagues have analyzed the early neutralizing antibody response in human patients to the alphavirus Chikungunya. Using a pooled set of 30 serum samples from Chikungunya virus-infected patients, the authors demonstrate that the early immune response is dominated by IgG3 antibodies directed against a single linear epitope found at the N-terminal end of the E2 glycoprotein. Removal of these antibodies from the serum resulted in a decrease in the Chikungunya-specific antibody titer. The value of this epitope as a potential diagnostic marker was demonstrated in nonhuman primates as well as in different patient cohorts. The study suggests that this epitope may prove useful in diagnostics as well as in the design of Chikungunya virus vaccines.

Response: We thank the referee for this supportive statement.

Specific comments:

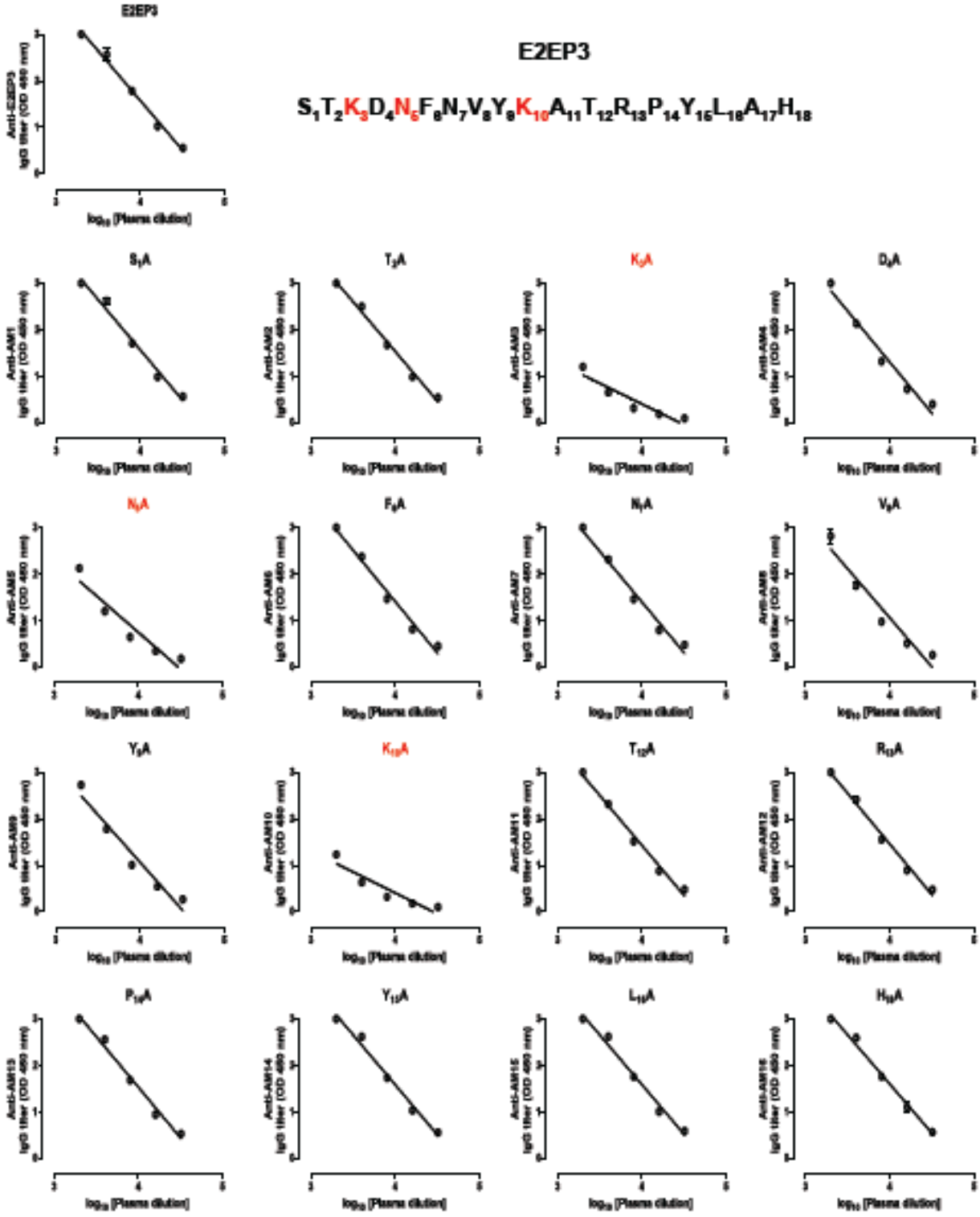
A major concern running through some of the manuscript is the lack of appropriate controls. This is highlighted in the experiments shown in figures 5, 7, and 8. The authors need to demonstrate the use of control peptides such as a randomized E2EP3 peptide and a peptide from region of E2 that had little or no reactivity with the sera as shown in Figure 2.

Response: The referee raised a valuable and important point. We have seriously considered all suggestions to strengthen the message of this manuscript. We have now repeated several key experiments that included two more control peptides from other regions of E2 that showed no reactivity with the patients' plasma shown in Figure 2, but have also tested them against the non-human primate plasma shown in Figure 7 and with mouse sera (Figure 8). The new data is now presented in the new Supplementary Figure 2 and mentioned in the text on lines 8 to 9 on page 9, line 11, page 12, and line 1 on page 13. A copy of the figure is shown below.



Supplementary Figure 2. Specificity of E2EP3. Individual peptides were screened using **A**. CHIKV-infected patient plasma pools (Median 10 days pio); **B**. CHIKV-infected non-human primates (NHP) plasma (13 days post-infection); and **C**. CHIKV-infected mouse sera (21 days post-infection). Black solid line represents the mean value of the healthy donors and controls. Dotted line represents the value of mean ± 6 SD. Results represent an average of 3 independent experiments. P6-1 represents the peptide sequence residing in E2 domain B, while P11-4 represents the peptide sequence residing in C-terminus of E2 glycoprotein (structure not resolved).

We have also discussed and debated closely with several co-authors on the necessity of getting the randomized E2EP3 control peptide as we already have the 16 E2EP3 control peptides with alanine substitutions. These 16 control peptides in fact are 'closer' to E2EP3 both in terms of sequence and structure than will be any scrambled peptides, and would act as more stringent and appropriate negative controls. In addition, the reactivity of all the 16 control peptides was also demonstrated as shown in the new Supplementary Figure 3 below.



Supplementary Figure 3. Alanine-scan analysis of E2EP3 by anti-CHIKV antibodies. Alanine substitutions were constructed at each position of E2EP3 except the existing alanines. CHIKV-infected patients' plasma pools were used to validate binding capacity. Plasma pools at median 10 days pio were tested in a set of serial dilutions from 1:2000 to 1:32000 and assayed in triplicates. Results are expressed as mean ± SD. Data are representative of 3 independent experiments.

Moreover, the control peptides containing the alanine substitutions that abrogated complete binding (Figure 5) had a sequence identity of 15 out of 18 amino acids (84%), further demonstrating the

exquisite specificity of anti-E2EP3 antibodies. Therefore, we feel that with these data, the use of a scrambled E2EP3 will have little benefits.

Page 13, line 1 (Fig 8A): Using the E2EP3 peptide to immunize mice only produced a modest reduction in infectivity of Chikungunya as measured in vitro. The authors should discuss how this compares with other immunogens and their ability to generate neutralizing antibodies.

Response: We thank the referee for this comment. As stated in the manuscript, we used an immunogen formulation where the E2EP3 peptide is coupled to KLH. This is a standard formulation to induce antibodies against peptides. In this study, this approach certainly did induce neutralizing antibodies as shown (Figure 8). However, it is true as stated by the referee that the *in vitro* activities of the antibodies induced was modest. From this study, now that we know the target epitope/antigen, we will be able to improve the design of the pre-clinical vaccine using multiple antigenic peptides (MAPs), recombinant proteins or even Virus-like particles (VLPs). These formulations have the advantage as CHIKV T helper epitopes could also be incorporated to provide help for efficient antibody production. To address this point by the referee, we have now discussed this in the manuscript on lines 6 to 9, page 16.

Page 14, line 12: The two sentences here are incongruent with one another. In the first sentence the authors refer to E2 as the only one of the three surface proteins that reacted with patient sera. In the second they say that neither capsid nor E1 proteins reacted in Westerns. However, capsid is not the third of the surface proteins and thus these two lines do not follow one another, or at the very least are confusing.

Response: We thank the referee for highlighting this and we apologize for the oversight. We have now amended the sentence to "…E2 glycoprotein was the only surface protein that reacted to the IgG of the patients' plasma collected during the early convalescent phase." on lines 15 to 17, page 14 to avoid any confusion. The second sentence has now been removed to improve the clarity.

Referee #2:

(Comments on Novelty/Model System):

The study Kam et al present is complete and elegant. The models used are the appropriate and the findings clear.

Response: We are very grateful to the referee for this positive comment on the quality of our manuscript.

(Other Remarks):

In their manuscript Kam et. al. present novel findings concerning the adaptive immune response against a medically important arbovirus, Chikungunya (CHIKV). Their study is complete and thorough and in my opinion promotes the field since it is adding new knowledge on the antibody response mechanisms against CHIKV, an area poorly understood.

Response: We wish to thank the referee once again for the positive statements.

I only have minor points, which I would however like to be addressed in the manuscript. These are:

1. In the title Early neutralizing IgG3 I believe 3 should be removed. The authors explain that in detail in the manuscript.

Response: We have now amended the title as recommended and the title now reads "Early neutralizing IgG response to Chikungunya virus in infected patients targets a dominant linear epitope on the E2 glycoprotein".

2. In the abstract, E2EP3-specific antibodies are neutralizing and their removal....up to 80%. This sentence is incomplete I think the word antibody should appear after CHIKV-specific.

Response: We apologize for this oversight and we wish to thank the referee for pointing this out. The sentence has now been amended accordingly with the word "antibody" added right after CHIKV-specific on line 11, page 3.

3. Page 6: Furin E2/E3-cleavage site that...the verb is missing here.

Response: We wish to apologize for this and the sentence has now been amended to "… furin E2/E3-cleavage site that is conserved in many…" on line 15, page 6.

4. Page 10: between parentheses E2EP3-specific IgG3 was; I believe this should read were to be in accordance with the tense used in the first sentence of the 3rd paragraph of page 15,

Response: We thank the referee for pointing this out to improve the clarity of the manuscript. We have amended the sentence accordingly on line 12, page 10 to "antibodies to E2EP3 were strongly neutralizing."

5. Page 15: We have previously showed; the verb should be changed to shown. Also since the article they are referring to is not yet published the word previously is not valid.

Response: We thank the referee for this suggestion and we have now changed the sentence to "We have shown" that early anti-CHIKV IgG3 …" on line 19, page 15.

6. Page 16: The authors discuss that early detection would allow more cost-effective patient management. I believe this is not the case since: a) they detect antibody at day 10 after the onset of symptoms when infectious virus is no longer detectable and b) the treatment for CHIKV is very similar to this used for other arboviruses and aiming only to relieve the symptoms.

Response: We have now removed this sentence as recommended by the referee on page 16.

7. Page 24: Animals were bled and observed daily for one week than...This should read then.

Response: We wish to sincerely apologize for this oversight and thank the referee for highlighting this. Sentence has now been corrected on line 6, page 22.

8. Figure 1. panel B is not required the western blots in both Figure 1 and Figure S1 are very clear.

Response: We thank the referee for raising this important point. Indeed, massive efforts were put in to assess the reactivity with western blot from all patients shown in Figure 1. Panel A is a representative of one single CHIKV-infected patient, and although the detection profiles were further validated by those shown in Figure S1, we felt that panel B is important as this further confirms that the observation shown in panel A also holds true for all other patients used in this study. Therefore, we believe that panel B is important to address this point and we hope that the referee will feel likewise.

9. Figure 5 panel D, when antibodies were depleted infectivity of the virus went only up to approx 50%. Could the authors comment on that?

Response: The referee has raised an interesting point. Although the response is directed mainly to a single linear epitope, we can't exclude that antibodies recognizing conformational epitopes are also present in patients' plasma. These antibodies wouldn't be depleted after incubation with the linear E2E3P peptides and therefore could still possibly mediate neutralization.

Referee #3:

(Comments on Novelty/Model System):

two appropriate and complementary models were used: human convalescent sera and murine infections

(Other Remarks):

In this manuscript, Lisa Ng's lab and collaborators have studied blood samples from ~30 convalescent people following the mosquito-borne Chikungunya virus infection. They determined that neutralizing antibody rapidly appears, and that this is of the IgG3 subclass. Further, they mapped the neutralizing epitope to an N-terminal conserved linear superficial peptide of the E2 glycoprotein (which they called E2EP3 stkdfnvykatrpylah), located the peptide on the 3D structure of the E2 glycoprotein. They determined crucial amino acids for the Ab binding of the peptide. Then, they extended the study by immunizing mice with virus and elicited anti-E2EP3 peptide Abs and finally vaccinated mice with the peptide-KLH, producing protection from arthritis (ankle swelling) following experimental infection.

The work is carefully and thoroughly done, presented in an elegant and compelling manner. It is highly likely this study will result in the development of a vaccine for people in the endemic Indian Ocean region, since eradication of the vector is impossible.

Response: We wish to thank the referee for the positive and supportive comments to our manuscript.

I would recommend one very minor modification to the manuscript: IgG3 is one of 2 Ab subclasses that are readily transmitted across the placenta. The paper's authors could add one sentence about the protection of newborns from infection via maternal immunization.

Response: We thank the referee for this suggestion and we have now included this description "Moreover, IgG3 is one of the two antibody subclasses that can be readily transmitted across the placenta (Palmeira et al, 2012), further suggesting that protection of newborns from CHIKV infections can occur via maternal immunization (Englund, 2007; Gerardin et al, 2008)." on lines 7 to 11, page 14.

3rd Editorial Decision 04 January 2012

Please find enclosed the final report on your manuscript.

We are pleased to inform you that your manuscript is accepted for publication and will be sent to our publisher to be included in the next available issue of EMBO Molecular Medicine if or once we have received your page charges form (pls. see below) .

Please also see below for additional IMPORTANT information and instructions regarding your article, its publication, and the production process.

Congratulations on your interesting work.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

The work here has been well done and presented. The relevance is that the authors examine a human cohort to identify the dominant epitope that the neutralizing response is directed at. The potential for using this epitope as a diagnostic and possibly a subunit vaccine is significant.

Referee #1 (Other Remarks):

This is a revised manuscript from Lisa Ng's laboratory examining the IgG response to Chikungunya

virus in infected human patients. The authors have modified the text appropriately in response to reviewer comments including an analysis of the use of control peptides to validate some of their assays. The manuscript, figures and supplementary information is now of sufficient quality and rigor for publication.

